Isolation and Characterization of Keratinase Producer Microbes from the Poultry Waste Material

SYNOPSIS

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Ph.D. Microbiology

Submitted by

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Introduction

Keratinases are proteolytic enzymes capable of degrading rigid and insoluble keratinous proteins present in skin and appendages (Adelere & Lateef, 2016). They are produced in the keratinous substrates such as feather, hair, wool, nail, horn and hoof by microorganisms (Vidmar & Vodovnik, 2018). For Keratinase production, thus the current research topic is therefore carried out to isolate Keratinase-producing bacteria and fungi from poultry waste feather and also determine the effect of cultural conditions on Keratinase production and feather degradation and to determine the effect of other substrates on Keratinase production. Feather produced in large quantities as by-products of poultry processing industries are the largest reservoirs of keratin (Godheja, Shekar & Modi, 2014). Keratinase is a hopeful mode in different biotechnological applications like as bioconversion of keratin wastes into animal feed and nitrogenous biofertilizer, keratinase is useful in cosmetic industries, medical pharmaceutical industries, textile manufacturing industries and leather industries (Tran et al., 2016). They are also applicable in the degradation of prion proteins, as pesticides and in the production of nanoparticles, biofuel, biodegradable films, glues and foils (Revathi et al., 2013). Researches into the microbial manufacture of Keratinases have been fast in the last decade, partially because of their ubiquitous production, and several potentiality applications in multitudinous areas of human endeavours. They also have potential usage in biomedical, leather, cosmetic, textile, and detergent industries (Adelere & Lateef, 2016).

The production of microbial Keratinase is less expensive than conventionally produced Keratinase. Microbial Keratinase can be obtained from some species of fungi, bacteria, and actinomycetes (Gopinath et al., 2015). There are few conventional methods for the treatment of feather wastes, such as burning of feather, land filling, steam pressure cooking of feather, and strong acid or alkali hydrolysis which is not only cause environmental pollutions, but also require large amount of energy for processing results in destruction of some essential amino acids (Yusuf et al., 2016). Keratin is a family of fibrous structural proteins, the monomers assemble into bundles to form intermediate filaments, which are tough and insoluble and form strong unmineralized tissues found in reptiles, birds, amphibians and mammals. They are biological tough materials only by chitin (Wakil, Dada & Onilude, 2011). Bacterial Keratinases are of peculiar interest

because of their function on insoluble keratin substrates and commonly on a wide range of protein substrates. These enzymes have been studied for de-hairing processes in the leather industry and hydrolysis of feather and keratin (Tork, Aly & Nawar, 2010).

The aims of this study is to isolate and identify a new efficient feather-degrading bacterium, which uses feather as carbon and nitrogen sources, from poultry decomposed wastes, also aimed to extraction, isolation, purification and characterization of Keratinase enzyme from the isolate. Keratin is an insoluble protein that is exist in nature principally in the form of feathers, hooves, wool, scales, nails (hard keratins) and stratum corneum (soft keratins) (Feroz et al., 2020). Proteins belong to the scleroprotein group are compounds which are highly resistant to the action of chemical, physical, and biological agents. They have high mechanical stability and high resistant to the degradation of keratin due to the presence of their disulfide bonds, hydrogen bonds, salt linkages and cross linkings (Deivasigamani & Alagappam, 2008). Keratin including materials are plenteous in nature but have insufficient uses in practices, meantime they are insoluble and resistant to degradation, but with the help of common proteolytic enzyme Keratinase the keratin wastes converted into valuable protein and amino acid (Narayanapp, Narayanapp &Mariswamy, 2019). Hair is mainly composed of 90% of keratin protein and 1-9 % of lipids (Avan et al., 2019).

The present research therefore is carried out to isolate Keratinase-producing bacteria from poultry feather waste, determine the effect of cultural conditions on Keratinase production and feather degradation and to determine the effect of other substrates on Keratinase production. Tannery industries are continually utilizing high amount of chemicals for dehairing in leather processing. These dehairing chemicals contain a high amount of pollutants such as soda-lime, salts, chromate, sulfide, solvent flashy, emulsified fatty matter, waste lime liquor. Beside these biological wastes from leather it causes environmental pollution (Barman et al., 2017). The chemicals utilized in leather processing industries are also caustic and health hazardous. Few tanneries have been compulsively close down due to their pollution impact in environment. Hereby, microbial and biotechnological perspective could be a promising and superior alternative to keratin and keratinous wastes recycling by enzymatic action by utilizing exclusive enzyme of microorganism named Keratinase. The potential applications of such microbial Keratinases have been reported (Barman et al., 2017). The hydrolysis of keratinous proteins i.e. amino acids used as animal feed and several other applications.

Literature Review

(Wakil, Dada & Onilude, 2011) have studied isolation and characterization of Keratinase-producing bacteria from poultry waste. They investigated that six feather-degrading bacteria were isolated from poultry waste and the optimum conditions for Keratinase synthesis by these isolates were determined, which was an essential step for the production of sufficient amounts for application in research. They maintained the pH of 7.2 that favour the production of Keratinase in most of the isolates. The optimum incubation time ranged from 48 hr to 96 hr and the optimum shaking speed of 150-250 rpm is optimum for Keratinase production.

(Bhuyar et al., 2018) have reported the isolation, characterization and partial purification of Keratinase from Keratinolytic bacteria. The utilization of purified enzymes for producing a useful product constitutes enzyme technology. Enzymes have a wide variety of applications in industry, medicine research etc. certain out of this one industrially crucial enzyme is Keratinase. It was an extracellular and proteolytic enzyme.

(Raja & Divakar, 2013) has been undertaken for the screening and isolation of Keratinase producing strains of bacteria from ten soil samples, collected from various regions of Bangalore and used to screen for Keratinase production by using feather powder agar plate assay.

(Barman et al., 2017) have reported the production, partial optimization and characterization of Keratinase enzyme by *Arthrobacter sp.* NFH5 isolated from soil samples Therefore, *Arthrobacter sp*. NFH5 can be used for large scale production of Keratinase for industrial purposes in short time.

(Narayanapp, Narayanapp & Mariswamy, 2019) have concluded the production and purification of Keratinase Enzyme from *Serratia sp.* isolated from poultry wastes. It revealed that *Serratia sp.* could be a potential microbial source can be utilize for dispense of environmental pollution and feather waste utilization.

(Raja & Divakar, 2013**)** have studied the screening and isolation of Keratinase producing bacteria from poultry waste. The organisms were tested for various biochemical tests, which leads to their identification as *Bacillus licheniformis, Bacillus aureus* and *Staphylococcus aureus*.

(Sahni, Sahota & Phutela, 2015) have reported the bacterial Keratinase and their prospective applications: a review. Keratinase were produced by species of *Bacillus, Streptomyces, Aspergillus, Fervidobacterium, Xanthomonas, Chryseobacterium* and *Vibrio.* Keratinase were useful in enzymatic de-hairing in leather and cosmetic industry .

(Desai et al., 2010) have studied the isolation of Keratinase from bacterial isolates of poultry soil for the degradation of waste. Here upon, the enzyme could retouch the poultry-processing waste containing keratin and have potential for biotechnological processing involving keratin hydrolysis.

(Pandian Sundaram & Panchatcharam, 2012) have concluded the isolation, identification and characterization of feather degrading bacteria. The protein profile of *Bacillus sp.* analyzed in SDS-PAGE. It showed that the presence of single band which corresponds to Keratinase activity.

(Balakrishnan & Padmanabhan, 2019) Identification and phylogenetic analysis of Keratinase producing bacteria SNP1 from poultry field, have concluded that the hydrolysis of a feather by *Bacillus haynesii* confirm that the bacterium has a substantial Keratinase activity and can break keratin disulphide bonds. Measuring enzymatic activity in seven isolated strains showed that *Bacillus* strain may be the best candidate in various industries.

(Purchase, 2016) Keratinase were versatile and valuable enzymes that degrade keratins and similar recalcitrant proteins. Increased consciousness of their biotechnological applications and potential has provided strong impetus to study this group of alkaline proteases.

(Mazotto et al., 2011) Three *Bacillus* species (*Bacillus subtilis* LFB-FIOCRUZ 1270, *Bacillus subtilis* LFB-FIOCRUZ 1273 and *Bacillus licheniformis* LFB-FIOCRUZ 1274), isolated from the poultry industry evaluated for Keratinase production by utilizing feathers as the sole carbon and nitrogen sources in a submerged fermentation. The three strains produced serine peptidases and shows Keratinase and gelatinase activity. *Bacillus subtilis* 1273 was the strain which exhibited the highest enzymatic activity.

(Selvam, Vishnupriya &Yamuna, 2013) have concluded that the Keratinolytic characters of crude enzymes checked by feather keratin as substrate and the protein concentration

was determined. Then, the isolates were identified at molecular level by 16S r-RNA gene amplification technique.

Research Gap Identification

A numbers of research group have reported for isolation of soil microbes but a few research literatures was available on isolation and characterization of Keratinase producer microbes from poultry waste soil. It is also found from survey that less research work has been done on isolation of Keratinase enzyme from the microbes. The present research therefore is carried out to isolate Keratinase-producing bacteria from poultry feather waste, determine the effect of cultural Keratinase on feather degradation.

So, the current research work is devoted for isolation of Keratinase producer microbes and their enzymes.

Objectives

1. Isolation, identification and characterization of Keratinolytic microorganisms
2. *In- vitro* screening of keratinolytic microorganisms
3. Purification of keratinase
4. Molecular characterization of best selected Keratinase producer microbes by 16s r-RNA amplification and sequencing
5. Application of purified keratinase enzyme

Proposed Methodology

Collection of poultry waste soil sample

The poultry waste soil sample will be collected from the dumping site of the poultry industry. The soil samples will be collected from the various feather dumping sites. Soil samples collected from 3 to 4 cm depth and transferred in sterile plastic bags by sterile spatula. The samples will bring to the Microbiology Research Laboratory for further study (Subugade, Gupta & Mokashe, 2017).

Isolation of Keratinase producer microbes

Isolation of microbes will be performed by serial dilution and plating method on Nutrient agar medium. One gram of soil sample transferred in 10 ml of sterilized distilled

water and mixed properly. Serial dilution made upto 10^{-6}. 0.1 ml of the diluted sample will inoculate in the nutrient agar medium plates from each dilution. The petri plates rotated clockwise and anticlockwise direction to disperse the sample equally. Incubate the plates at 37˚C for 24-48 hrs. The bacterial isolates further sub cultured on nutrient agar medium to obtain pure culture. Pure culture of isolates maintained in nutrient agar media slants at 4˚C for further studies (Kumar, Kartik & KV, 2010).

Screening of Keratinolytic microbes

1. Screening of Keratinase production by plate assay

The isolates screened for Keratinase activity. This will do by inoculating the microorganisms on the feather powder agar plates containing 2% feather powder. For this the chicken feathers washed and dried at 50˚C in an oven, the dried feathers ground into fine fractions. Incubate the plates at 37^{o}C for 48 hrs. (Subugade, Gupta & Mokasha, 2017).

2. Secondary Screening of Keratinolytic Bacteria

The selected protease producing bacteria subsequently grown in feathers consisted of (0.5 gm) NaCl, (0.1 gm) $MgCl_2.6H_2O$, (0.06gm) $CaCl_2$, (0.7gm) KH_2PO_4, (1.4 gm) K_2HPO_4, and (1g) feather, pH of the medium adjusted to 7.5 (using 1 N NaOH). Cultures will grown at 37°C at 120 rpm for 96 hrs. Keratinolytic strain that completely broke down feathers in the medium selected for further study (Subugade, Gupta & Mokasha, 2017).

Morphological Identification of Keratinolytic Bacteria

The isolates will be observed under the microscope, the colony morphology will noted with respect to shape, size, margin of colony and pigmentation (Divyasree et al., 2018).

1. Microscopic Observation

Bacterial isolates Gram stained and observed under a high power magnifying lens in light microscope (Divyasree et al., 2018).

Biochemical characterization of selected Keratinase producer microbes

1. Biochemical Characterization

The bacterial isolates characterized biochemically by Indole test, Methyl red test, Vogus proskaur's test, Citrate utilization test and Carbohydrates test (Jani et al., 2014).

Optimization of hyper Keratinase producer microbes

1. Effect of pH

Nutrient broth medium containing 0.1% feather prepared at different pH 4, 6, 8 and 10. The bacterial isolate will inoculate in to the nutrient broth medium. Inoculated mediums incubated at 37ºC for 48 hrs. Absorbance of the medium will have to be measured using spectrophotometer at 590 nm against the nutrient broth as blank (Tamilmani et al., 2008).

2. Effect of temperature

Nutrient broth medium prepare in conical flasks and conical flasks autoclaved and the bacterial cultures inoculate into the Nutrient broth medium containing 1% feather. The pH of each conical flask adjusted to 7. The flasks will be incubate at different temperature like, 25, 35, 45, and 55˚C. After 72 hrs of incubation, the absorbance of the medium will have to be measured using spectrophotometer at 590 nm against the nutrient broth as blank (Tamilmani et al., 2008).

3. Effect of metal ions

About 50 ml of broth with 1% of feather will be prepared in four different conical flask and 10 mg of each metal ions like cupric sulphate, cadmium sulphate, manganese chloride and zinc sulphate used. Autoclaved the media, microorganism inoculated to each conical flask and incubate at 35˚C for 72 hrs. Afterwards, keratinolytic activity will measured by using spectrophotometer (Narayanapp & Mariswamy, 2019).

Enzyme Production

The selected isolates after identification and characterization inoculated into the feather basal medium and incubated on shaker incubator at 35°C for 7 days (Bhuyar et al., 2018).

Filtration

The culture medium filtered via Whatmann No.1 filter paper to remove undegraded residues.

Centrifugation

After incubation the broth will collected and centrifuged at 10,000 rpm for 10 mins. Supernatant containing the extra cellular secretions and pellet to be discarded (Bhuyar et al., 2018).

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Partial purification of Keratinase from selected isolates

About 200 ml Keratinase broth prepared for the purification of enzyme. Cell free media will use for the purification by ammonium sulphate salt followed by centrifugation.

1. Ammonium sulphate precipitation

70 ml of supernatant will be used for the precipitation with 80% ammonium sulphate. All subsequent steps are carried out at 4°C. The resulting precipitate will be obtained by centrifugation at 10000 rpm for 10 minutes. The precipitate dissolves in 50 mM phosphate buffer.

2. Molecular weight determination of isolated Keratinase enzyme

Polyacrylamide gel electrophoresis (PAGE) in presence of Sodium Dodecyl Sulphate (SDS) will be prepared. About 40 µL of the sample mixed with 40 µL of gel loading dye, 50 µL of sample will have to be loaded into wells. The necessary electrical connections will made and the voltage adjusted to 50 mV. Electrophoresis will made to run for 75-80% of the length of the gel slab. Gel will stained with coomassie blue for 8 hrs and destines to observe the bands (Tork, Aly & Nawar, 2010).

Molecular characterization of best selected Keratinase producer microbes by 16s r-RNA amplification and sequencing

Genomic DNA will be extracted according to the standard procedure. The 16s r-RNA of the strains amplified using the 16s r-RNA primers, fD1 (5'-GAGTTTGATCCTGGCTCAG-3') and Rp2 (5'-ACGGCTACCTTGTTACGACTT-3'). PCR products purified using QIA quick PCR purification kit. The purified DNA fragments will be sequenced using sequencer model ABI 3100 sequencer according to the protocol provided by the manufacturer (ABI PRISM 3100 Genetic Analyzer User's Manual) (Maidak et al.,2000).

Phylogenetic analysis

16s r-RNA sequence analysis performed with the aid of molecular evolutionary genetic analysis by using 4 software package neighbor-joining method (Chen et al., 2021). For the phylogenetic trees, only sequences from the type strains whose names have been validly published taken into account.

Application of purified Keratinase enzyme

Production of nutrient rich compost

Poultry waste materials will be recycled with the application of purified keratinase to produce nutrient rich compost which leads to increase soil fertility without any harmful effect on soil biota (Verma et al., 2017).

Thesis Outline

Chapter 1 (Introduction**):** It will include general information about the proteolytic enzyme keratinase and non biodegradable material keratin. It will also include objectives of the study and research gaps that originated the need for the present study.

Chapter 2 (Literature Review**):** This chapter will include the deep review of the literature concerning old and latest finding of various researchers regarding the isolation of keratinase producer microbes and application of keratinase.

Chapter 3 (Materials and Methods)**:** This chapter contains the Research Methodology. Methodology of sample collection, isolation and characterization of keratinase producer microbes, screening of keratinolytic activities *in-vitro* conditions and molecular characterization of best selected Keratinase producer microbes by 16s r-RNA amplification and sequencing, lab work, lab observation.

Chapter 4 (Results and Discussion)**:** This chapter will be described the results of Qualitative Screening of keratinase production, Morphological Identification of Keratinolytic Bacteria and phylogenic analysis of isolated keratinase producer microbes.

Chapter 5 (Summary and Conclusions)**:-**This chapter will comprise the conclusion of whole study based on objectives along with summary. The scope of further research will also be discussed.

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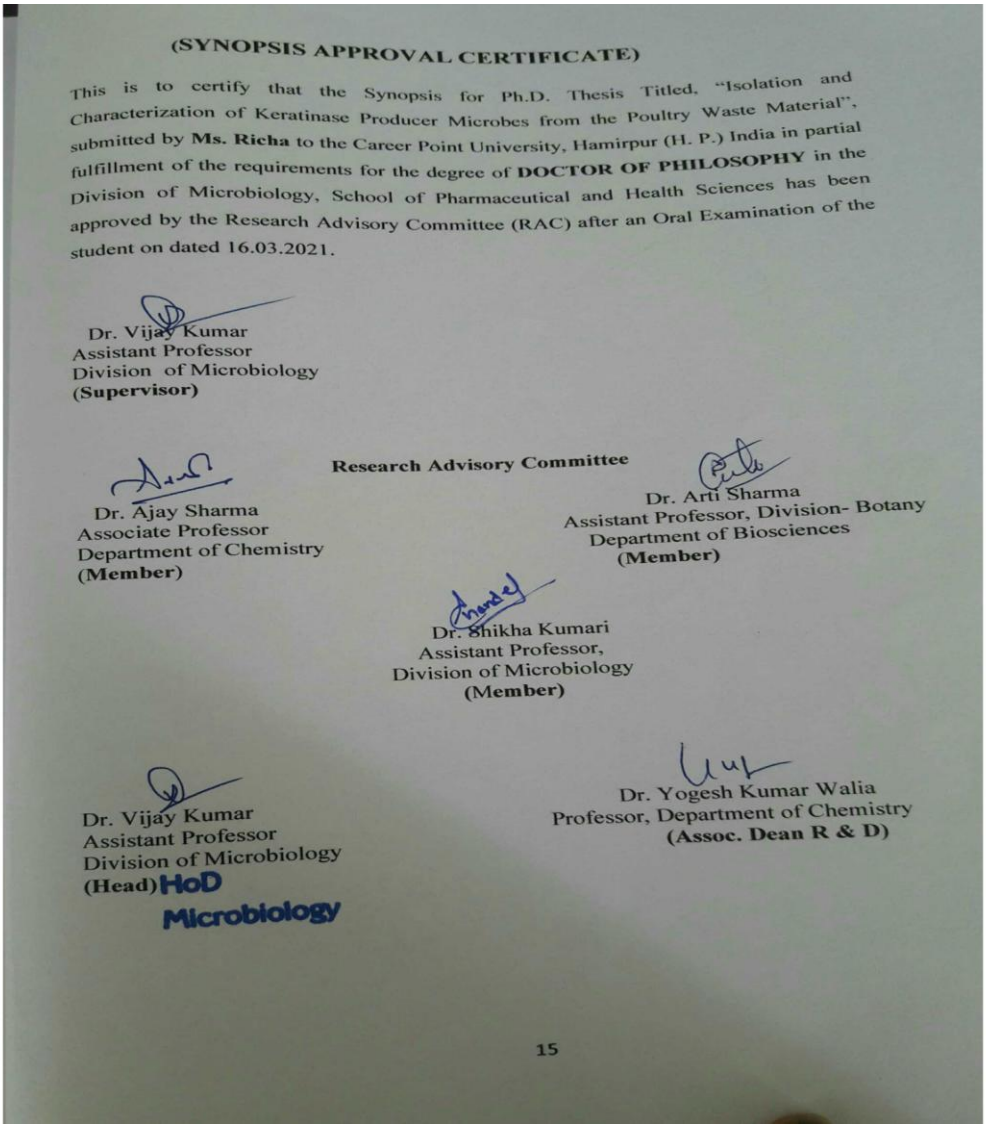

(SYNOPSIS APPROVAL CERTIFICATE)

This is to certify that the Synopsis for Ph.D. Thesis Titled, "Isolation and Characterization of Keratinase Producer Microbes from the Poultry Waste Material", submitted by **Ms. Richa** to the Career Point University, Hamirpur (H. P.) India in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in the Division of Microbiology, School of Pharmaceutical and Health Sciences has been approved by the Research Advisory Committee (RAC) after an Oral Examination of the student on dated 16.03.2021.

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